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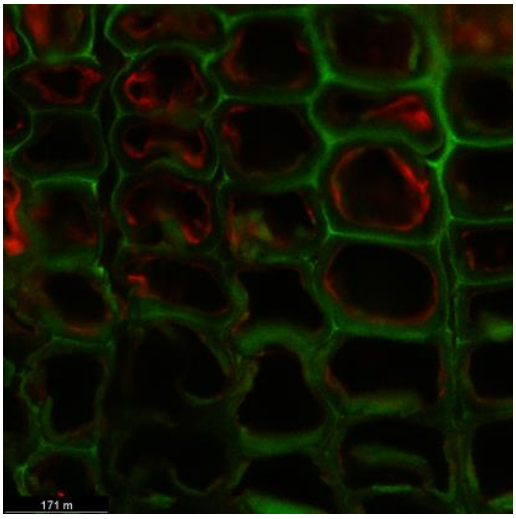
INITIATION OF LIGNIFICATION IN NORWAY SPRUCE XYLEM DETECTED BY IMMUNOLABELING AND RAMAN SPECTROSCOPY



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Wood development is a significant process with both financial as well as natural perspectives. Trees and wood are of highly significance in Finland where a huge part of the gross national income devises from the forestry area. Ecologically and commercially the Norway spruce (Picea abies) is one of the most common tree species in Europe. It covers about 30% of Finland's forest area. Norway spruce is frequently used in research to study many phenomena related specifically to the wood formation and lignification.

The principal objective of my thesis work was to reveal an unknown step in the lignification process in developing xylem of Norway spruce, i.e. the initiation site(s) for lignification. To achieve this goal, the aim was to investigate the chemical identity of possible lignification initiation sites in the middle lamellae and cell corners of developing Norway spruce xylem, and to answer the question where in the cell wall soluble monolignols first emerge and lead to the start of lignin formation (polymerization).

I was approaching this goal with immunolabeling technique for confocal microscopy and Raman spectroscopy to unravel this initiation site of lignification by using specific monoclonal antibodies for cell wall compounds and comparing the results with the initial lignin deposition sites.

To detect the location/distribution of some important polysaccharides and lignin substructure for lignification initiation, monoclonal antibodies i.e. LM10, LM11, LM15, LM24 and antibody Dibenzodioxocin or DBD were applied for confocal microscopy and some monolignol specific spectra were applied for Raman microscopy. The xylan was detected by LM10 in secondary cell wall abundantly and few are in primary cell wall of Norway spruce. The LM11 against arabinoxylan was determined more in primary cell walls but less in secondary cell wall. The location of xyloglucan was identified in the middle lamellae, primary and secondary cell wall of Norway spruce by LM15. The LM24 against glycosylated xyloglucan was found in secondary cell walls, abundantly in cell corners but few in primary cell wall. The primary antibody Dibenzodioxocin or DBD for the lignin substructure revealed that these were present in the mature cells of secondary cell walls (S2 and S3 layers). The lignin substructures DBD were not found in youngest cells where secondary cell walls are absent.

The developing xylem of Norway spruce was subjected Raman microscopy and which revealed the locations of cinnamyl alcohol, coniferyl alcohol and coniferyl aldehyde. The cinnamyl alcohol was abundantly found at cell corner and middle lamellae in most developing part of xylem. The coniferyl alcohol was determined only in developing xylem cell corners. The coniferyl aldehyde was observed at cell corners, middle lamella and primary cell walls of developing xylem. The coniferyl aldehyde was located more in mature cells than younger cells.

So, the Confocal and Raman microscopy images revealed the possible bindings of monolignols to polysaccharide in young cell corners, cell wall layers and middle lamellae.

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DEDICATION

This thesis is dedicated to all of my family, particularly to my mother Sajeda Begum and brother Mohammad Shafiqul Islam Bhuiyan and my uncle Md. Sohrab Hossain and aunty Mst. Hasina Begum.

Al Amin

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LIST OF ABBREVIATIONS

OMT	O-methyltransferase
PAL	Phenylalanine Ammonia-lyase
CAD	Cinnamyl Alcohol Dehydrogenase
MFA	Microfibril Angle
AGPs	Arabinogalactan Proteins
HRGP	Hydroxyproline
PRPs	Proteins Rich in Proline
GRPs	Proteins Rich in Glycine
Hyp	Hydroxyproline
GT	Glycosyl transferases
RG	Rhamnogalacturonan
AGPs	Arabinogalactan proteins
XGA	Xylogalacturonan
DP	Degree of Polymerization
HG	Homogalacturonan
GB	Giga Base Pairs
EW	Earlywood
LW	Latewood
ABC	ATP-Binding Cassette
CA	Coniferyl Alcohol
SA	Ainapyl Alcohol
p-CA	p-coumaryl Alcohol
LUKE	Natural Resources Institute Finland
AIR	Alcohol Insoluble Residue
MAb	Monoclonal Antibody
CoMPP	Microarray Polymer Profiling
PBS	Phosphate Bbuffer Solution
DBD	Dibenzodioxocin

TABLE OF CONTENTS

ABSTRACT 2

DEDICATION 3

LIST OF ABBREVIATIONS 4

1 INTRODUCTION 7

1.1 Structure of plant cell wall 9

1.1.1 Lignin 13

1.1.2 Cellullose 14

1.1.3 Hemicellulose 16

1.1.3.1 Xylans 17

1.1.3.2 Xyloglucan 17

1.1.3.3 Arabinoxylan 18

1.1.4 Pectins 19

1.1.5 Proteins 20

1.1.6 Other polysaccharides 21

1.1.6.1 Xylogalacturonan 21

1.1.6.2 Homogalacturonan 21

1.2 Norway Spruce 22

1.2.1 Developmental biology of Norway spruce xylem 23

1.3 Lignification in plant cells 24

1.3.1 Lignification in developing xylem 26

2 HYPOTHESIS AND OBJECTIVES 27

3 MATERIALS AND METHODS 28

3.1 Methods of sample preparation 28

3.1.1 AIR procedure 29

3.1.2 Sample preparation for immunolabeling 29

3.2 Immunolabeling with monoclonal antibodies against plant cell wall compounds 30

3.3 Confocal Microscopy 31

3.4 Raman Microscopy 32

4 RESULTS 33

4.1 Confocal Microscopy 33

4.1.1 Detection of xylan with LM 10 antibody 33

4.1.2 Detection of arabinoxylan and unsubstituted xylans with LM11 antibody 34

4.1.3 Detection of XXXG motif in xyloglucan with LM15 antibody 36

4.1.4 Detection of galactosylated xyloglucan with LM24 antibody 37

4.1.5 Detection of dibenzodioxocin (DBD) with a rabbit antibody 38

4.2 Raman microscopy 39

5 DISCUSSION 41
6 CONCLUSIONS 43
7 ACKNOWLEDGEMENT 43
8 REFERENCES 43

1 Introduction

The three key chemical constituents of secondary xylem or wood are cellulose, hemi-cellulose, and lignin. Lignin is like glue which holds the fibers of cellulose together. Removal of lignin is very essential during wood pulping to get only pure fibers for paper making. Now-a-days tree genetics and biochemistry are research interests in forest science. The pulp yield can be improved either by changing in quality or reducing in the quantity of lignin in the trees which are grown for pulp and paper. The lignin biosynthesis pathway is well established. The enzymes encoding genes for lignin biosynthesis have been cloned from *Populus* and *Pinus*. For instance, O-methyltransferase (OMT) (Bugos et al., 1991; Li et al., 1997); phenylalanine ammonia-lyase (PAL) (Whetten & Sederoff, 1992; Subramaniam et al., 1993); cinnamyl alcohol dehydrogenase (CAD) (O'Malley et al., 1992; Van Doorsselaere et al., 1995), 4-coumarate-CoA ligase (4CL) (Voo et al., 1995; Zhang & Chiang, 1997; Allina et al., 1998) encoding genes were discovered successfully.

With the cloning approach of these genes, *in vivo* expression, natural variation and relationship to lignin content distinction in wood were studied. Genetic manipulation of 4CL and OMT genes in *Populus* demonstrates that alteration of these genes affect the quantity and quality of lignin. Hue and his team discovered that 4CL gene silencing in *Populus* led to 40-45% reduction in lignin content in the mutant (Hue et al., 2016). It was also discovered that reduced 4CL expression and declined lignin content resulted in increasing cellulose content and faster growth rates. There are several enzymatic steps to biosynthesis of phenylalanine and the three distinct lignin monomers. The enzymes of the pathways (C4H, PAL etc.) and the encoding genes have been cloned from various tree species and their expression patterns and structure are being investigated. The CAD controls the final enzymatic step for lignin monomers biosynthesis. In 1997, Mackay and his research team found that a *Pinus taeda* mutant had significantly reduced CAD expression and produced abnormal lignin. Such research results indicate the potential and difficulties for lignin modification in trees for pulp production.

Wood formation is a significant procedure with both natural and financial perspectives. Trees and wood are of extensive significance in Finland where a huge piece of the gross national income originates from the woodland area. Wood is a basic common and sustainable asset for

some human exercises for example in paper and pulp businesses, house development, and bioenergy generation. In addition trees offer potential for the creation of novel pharmaceuticals and fibers for garments in biorefineries. Norway spruce (*Picea abies*) is one of the most common tree species in Europe, both ecologically and commercially, covering 25-30% of Finland's forest area. Norway spruce is often used in research to study many phenomena related specifically to the formation of wood and lignin (Koutaniemi et al., 2007; Fagerstedt et al., 2010; Laitinen et al., 2017; Jokipii-Lukkari et al., 2018). Wood cell walls contain lignin, a phenolic biomolecule, up to 27-32 percent of dry material in the cell wall.

Lignin is the second most abundant biopolymer on Earth after cellulose (Misra et al., 2011) and it plays a crucial role for cell wall structural integrity and plant body stiffness and strength. It has a negative impact on the pulping operation, however, and its removal from pulp requires a huge cost. In addition, lignin is also one of the most important negative factors in the conversion of wood into biofuels. However, there is an emerging use of lignin for high-value plastics replacement products. Lignification is initiated in xylem cell walls in the cell corners and middle lamellae (Donaldson, 2001; Marjamaa et al., 2003), rich in pectic polysaccharides such as homogalacturonan with many calcium bridges (Bush & McCann, 1999). Results from earlier research have shown that there are three possibilities to connect polysaccharides to lignin.

1. Direct associations with either ether or ester bonds,
2. Linkages through ferulic acid residues, or
3. Dihydroferulic acid bridges between the polysaccharide and lignin (Iiyama et al., 1994; Ralph et al., 1998; Ralph & Landucci, 2010).

The primary cell wall of conifers (Carnachan & Harris, 2000) also contains small amounts of ester-linked ferulic acid residues, which could serve as nucleation sites for lignin polymerization (Ralph et al., 1994). In addition, several cell wall proteins are rich in tyrosine (Showalter, 1993; Domingo et al., 1994), which may form lignin cross-links. Adding tyrosine-rich peptides to transgenic hybrid poplar has resulted in increased release of lignin from protease treated xylem, suggesting lignin cross-linking to tyrosine residues (Liang et al., 2008). Therefore, if present in the middle lamellae, tyrosine-containing proteins may serve as nucleation sites for polymerization of lignin.

When monolignols are transported to the apoplast, they are activated by laccases and peroxidases to phenolic radicals, accompanied by polymerization to lignin (Fagerstedt et al., 2010; Kärkönen & Kuchitsu, 2015). Laccases use atmospheric oxygen for the oxidative power while peroxidases need hydrogen peroxide (H_2O_2) for this purpose. Such polymerization, however, begins at the location most remote from the site of the plasma membrane, i.e. in the middle lamellae and cell corners. It is not known what channels monolignols from the plasma membrane to the middle lamellae, and limits their reaction to the correct location, causing lignification initiation. The ester-linked residues of ferulic acid (Carnachan & Harris, 2000) or tyrosine residues present in the proteins of the cell wall near the middle lamellae and the primary cell wall may be the initiation sites of lignin formation in Norway spruce.

1.1 Structure of plant cell wall

The plant cell wall is a dynamic structure that fulfills a wide range of roles in the plant life cycle. The cell wall offers flexibility to promote cell division, a biochemical scaffold that allows differentiation, and a pathological and environmental barrier that defends against stress, in addition to preserving structural integrity by resisting internal hydrostatic pressures (Scheller & Ulvskov, 2010; Tucker & Koltunow, 2014). The cell wall contains a wide variety of receptors, channels and pores that monitor molecular movement and local and long-range elicitor responses, including sugars, hormones, RNAs and proteins. Plant cell wall structure is extremely varied, not only among plant species, but also between tissue types, consistent with a role in many processes. In general, the primary wall and secondary wall are sometimes referred to as two types of wall surrounding plant cells. During division, a dynamic primary wall is formed in young cells and acts to provide flexibility and basic structural support, protects the cell, and mediates interactions between cells. The thicker and more resilient secondary wall is formed between the primary wall and the plasma membrane, and it is deposited only after the cell has stopped dividing and expanding. The secondary wall is seen as a key adaptation that enables terrestrial plants to facilitate and withstand upright growth.

Cellulose, pectic and non-cellulosic polysaccharides, phenolic compounds, proteins, and water are common components of the cell wall. The key components (more than 90%) are

polysaccharides, whose structure and biosynthesis have recently been extensively examined (Atmodjo et al., 2013; Burton et al., 2010; Pauly et al., 2013; Rennie & Scheller, 2014; Kumar et al., 2016). In short, cellulose is a carbohydrate insoluble in water contained in both primary and secondary cell walls whose fibrous structure allows structural integrity to be preserved. Pectins, which are arguably the most heterogeneous and complex polysaccharides of the cell wall, reside predominantly in the primary cell wall and have expansion, porosity, strength, adhesion, as well as intercellular signaling functions. Xylan, xyloglucan, mannan, callose and β-1,3:1,4-glucan are other abundant non-cellulosic polysaccharides, which accomplish different roles in reserve storage, mechanical support and growth. In comparison to cellulose, sugar substitutions and side chains that are bound to the polysaccharide backbone during biosynthesis may further differentiate between pectic and non-cellulosic polysaccharides (Scheller & Ulvskov, 2010). The above mentioned substituents influence viscosity, solubility and interactions with proteins and other polysaccharides within the cell wall.

Generally, plant cell walls have three layers or strata i.e. primary cell wall, secondary cell wall and middle lamella.

- The primary cell wall is normally a flexible, thin as well as extensible layer when a cell is in the growing stage.
- Normally the secondary cell wall is a thick layer which is formed in fully grown cell inside the primary cell wall. It is not present in all plant cell types. For instances some cells like xylem possesses lignified secondary cell wall, which makes it waterproof and strengthens the wall.
- The pectins enriched middle lamella is the outermost stratum which glues the adjacent plant cells to each other and makes an interface between them.

In the primary plant cell wall the principal carbohydrates are cellulose, hemicellulose and pectin. The cellulose microfibrils are bound to form a cellulose-hemicellulose network mediated by hemicellulosic tethers that is embedded in the matrix of pectin. Xyloglucan is the most common hemicellulose in the primary cell wall. Xyloglucan and pectin are reduced in abundance in grass cell walls and partially replaced by glucuronarabinoxylan, another hemicellulose type. Characteristically, primary cell walls expand (grow) by a process called acid growth, facilitated by expansins, extracellular proteins activated by acidic conditions that change the pectin-

cellulose hydrogen bonds. This functions to increase the extensibility of the cell wall. The outer portion of the primary cell wall of epidermis cells is normally impregnated with wax and cutin, creating a permeability barrier known as the cuticle of the plant (Carpita & Gibeaut, 1993).

Cell wall composition varies between species and can depend on the type of cell and stage of growth. Polysaccharides cellulose, hemi-celluloses and pectins are components of the primary cell wall of terrestrial plants. Sometimes other polymers are anchored to or embedded in plant cell walls, such as lignin, cutin or suberin (Keegstra et al., 1973).

Situated between the primary cell wall and the plasma membrane, the secondary cell wall is a structure found in many plant cells. After the primary cell wall is mature and the cell has stopped growing, the cell begins developing the secondary cell wall. Secondary cell walls provide the larger plant with extra protection for cells and rigidity and strength. These walls are composed of layered sheaths of microfibrils of cellulose, wherein the fibers within each layer are parallel (Liepman et al., 2010). The lignin inclusion makes the secondary cell wall less water-permeable and less flexible than the primary cell wall. The hydrophobic nature of lignin within these tissues is important for the containment of water within the vascular tissues that carry it throughout the plant, in addition to making the walls more resistant to degradation. A wide variety of additional compounds contain secondary cell walls that change their mechanical properties and permeability. The secondary cell wall, along with other polysaccharides, lignin, and glycoprotein, consists primarily of cellulose. In general, wood (mainly secondary cell walls) is made up of major polymers i.e. cellulose (35-50%), xylan (a type of hemicellulose, 20-35%), and lignin, 10-25%, a complex phenolic polymer that penetrates the cell wall spaces between the components of cellulose, hemicellulose and pectin, pushing water out and reinforcing the wall (Li & Chapple, 2010).

There are often three different layers in the secondary cell wall - S1, S2 and S3 - where the orientation of the cellulose microfibrils varies between the layers (Buchanan et al., 2015). The direction of the microfibrils is referred to as the microfibril angle (MFA). A low microfibril angle is observed in the S2 layer in the secondary cell wall of tree fibers, while the S1 and S3 layers display a higher MFA. However, depending on the load on the tissue, the MFA may also adjust. The MFA in S2-layer can vary in reaction wood, it has been shown. Tension wood has a

low MFA, meaning that the microfibril is oriented parallel to the axis of the fibre, The MFA is strong in compression wood and reaches up to 45°. These differences affect the cell wall's mechanical properties (Donaldson, 2008).

Compared to the primary wall, the secondary cell wall has different ratios of constituents. An example of this is that there are polysaccharides called xylan in the secondary wood wall, while the primary wall contains xyloglucan. In secondary walls, the cellulose fraction is also higher (Taiz & Zeiger, 2006). Pectins can also be absent from the secondary wall, and no structural proteins or enzymes have been identified, unlike primary walls. As the permeability through the secondary cell wall is low, cellular transport is carried out through openings in the wall called pits.

Wood consists primarily of a secondary cell wall that holds the plant against gravity (Campbell, 2005). Some secondary cell walls, such as those in cotyledons and the endosperm, contain storage compounds. Little cellulose and mostly other polysaccharides are found in these. In order to withstand internal osmotic pressures of several times atmospheric pressure arising from the difference in solute concentration between the inner cell and external solutions, plant cell walls must have adequate tensile strength. Plant cell walls vary in thickness from 0.1 to several μm (Buchanan et al., 2015).

In addition, structural proteins (1-5 percent) are present in most cell walls of plants; they are known as glycoproteins rich in arabinogalactan proteins (AGP), hydroxyproline (HRGP), proteins rich in proline (PRPs) and proteins rich in glycine (GRPs). A characteristic, highly repetitive protein sequence distinguishes each of these classes of glycoproteins. Maximum cell wall proteins are glycosylated, contain hydroxyproline (Hyp) and in the cell wall they become cross-linked. In specialized cells and in cell corners, these proteins are often concentrated. Epidermis cell walls can contain cutin. In the endodermis roots, the Casparian strip and cork cells of plant bark produce suberin. Both suberin and cutin are polyesters that act as barriers to water movement by permeability. The relative composition of carbohydrates, proteins and secondary compounds varies between plants and between the form and age of the cells. The walls of plant cells also contain numerous enzymes that trim, cut and cross-link wall polymers, such as hydrolases, peroxidases, esterases, and transglycosylases. Secondary walls can also

contain microscopic silica crystals, especially in grasses, which strengthen the wall and can protect the plant from herbivores (Jamet et al., 2006). In certain plant tissues, cell walls also act as storage deposits for carbohydrates that can be broken down and reabsorbed to supply the plant's metabolic and growth needs. For instance, endosperm cell walls are rich in glucans and other polysaccharides in cereal grass seeds, nasturtium and other species, which are readily digested by enzymes during seed germination to form simple sugars that nourish the developing embryo (Somerville et al., 2004).

The middle lamellae is first laid down, developed during cytokinesis from the cell plate, and the primary cell wall is then deposited within the middle lamella. The actual molecular interactions in cell wall structure are not well described and there are several models - the covalently connected cross model, the tether model, the model of the diffuse layer and the model of the stratified layer. However, the primary cell wall can be defined as composed of cellulose microfibrils that are not aligned. Cellulose microfibrils are formed by the cellulose synthase complex on the plasma membrane, which is proposed to be made of a hexameric rosette containing three catalytic subunits of cellulose synthase for each of the six units (Rose et al., 2004). To provide a high tensile strength, microfibrils are held together by hydrogen bonds. The cells are kept together with a gelatinous membrane called and the middle lamella, containing magnesium and calcium pectates (salts of pectic acid). A secondary wall is formed between the plasma membrane and the primary wall in some plants and cell types after a maximum size or point of growth has been reached (Bertone & Snyder, 2005). Unlike in the primary wall, the cellulose microfibrils are aligned in parallel layers, the orientation varying slightly with each additional layer so that the structure becomes helicoidal (Rose et al., 2004). Like the gritty sclereid cells in pear and quince fruit, cells with secondary cell walls can be rigid. Cell to cell communication becomes possible through pits in the secondary cell wall, and these also allow plasmodesmata to link cells through the secondary cell walls.

1.1.1 Lignin

The word ‘Lignin’ was derived from the Latin word ‘lignum’, which means wood. It was coined by a Swiss botanist A. P. de Candolle in 1813. Chemically, it is a cross-linked phenolic polymer

(de Candolle, 1844). It is an essential structural material of support tissues of all vascular plants and some algae. Generally lignin polymers (Fig. 1) are comprised of p-hydroxyphenyl (H), and syringyl (S) guaiacyl (G) units which are derived from the three primary monolignols p-coumaryl, sinapyl and coniferyl alcohols. The lignin polymerization happens via radical coupling reactions, where enzymes are needed to catalyze oxidation of monolignols or lignin monomers (Boerjan, 2003). Lignin biosynthesis initiates in the cytosol with the synthesis of glycosylated monolignols from the phenylalanine amino acid. These initial reactions are shared with the phenylpropanoid pathway. The attached glucose units render them less toxic and water-soluble. The glucose units are removed when monolignols are transported through the cell membrane to the apoplast (the exact location of this glucose removal is not known), and the polymerisation initiates (Samuels et al., 2002). Lignin fills the blank spaces in the cell wall among hemicellulose, cellulose as well as pectin components. Covalent bonds between lignin and hemicellulose mediates the cross-link among different polysaccharides. Thus, it increases the strength of cell wall, and supports the plant as it extends. It is predominantly present in compression wood but limited in tension wood, which are sorts of reaction wood (Chabannes et al., 2001).

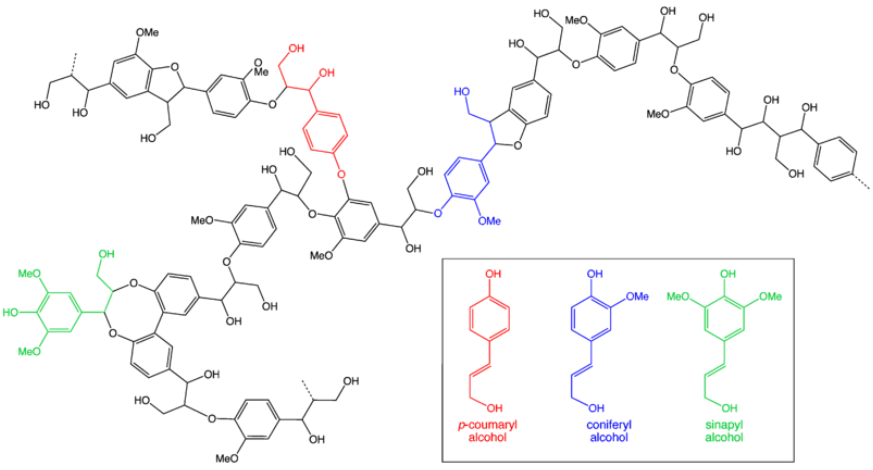

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Figure 1. The general structure of lignin. Lignin monomers are presented with colours.

1.1.2 Cellulose

The most ample organic polymer on the Earth is cellulose. The cellulose $(C_6H_{10}O_5)n$ is a polysaccharide which consists of many (several hundred to thousands) β(1→4) linked D-glucose

units (Figs 2 & 3). The D-glucose units are bound by glycosidic bonds and form a linear chain polymer. In 1838, cellulose was discovered by Anselme Payen (Crawford, 1981).

The biosynthesis of cellulose occurs at the plants plasma membrane by rosette terminal complexes (RTCs). The RTCs are hexameric proteins complexes which contain the cellulose synthase to produce cellulose chains. The RTC floats in the plasma membrane of cell and "twists" a microfibril into the cell wall. RTCs contain at least three distinct cellulose synthases, encoded by CesA (Ces is short form of "cellulose synthase") genes. In primary and secondary cell wall biosynthesis, separate sets of CesA genes are involved. In the plant CesA superfamily, there are considered to be about seven subfamilies, some of which include the more obscure, tentatively-named Csl (cellulose synthase-like) enzymes. To produce the β(1→4)-linked cellulose, these cellulose syntheses use cytosolic UDP-glucose (Kimura et al., 1999; Taylor, 2003; Richmond, 2000).

Bacterial cellulose is generated using the same protein class, although in many cases the gene is named BcsA for "bacterial cellulose synthase" or CelA for "cellulose". In fact, plants acquired CesA from the chloroplast-produced endosymbiosis event. All known cellulose synthases belong to the family of glucosyltransferase 2 (GT2) (Popper, 2011).

Cellulose synthesis requires two separate processes i.e. chain initiation and elongation. Cellulose synthase (CesA) starts cellulose polymerization by using a steroid primer, UDP-glucose, and sitosterol-beta-glucoside. Then it utilizes UDP-D-glucose precursors to extend the growing chain of cellulose (Peng et al., 2002; Barsett et al., 2005).

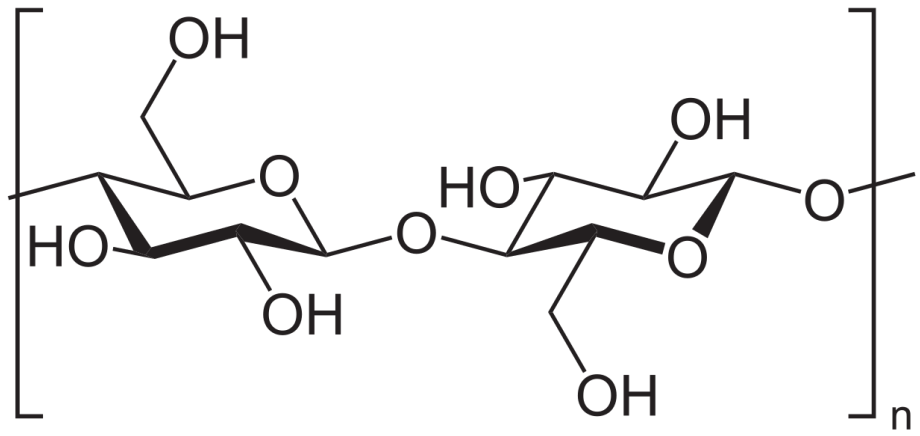

Figure 2. Cellulose, a linear polymer of D-glucose units (two are shown) linked by β(1→4)-glycosidic bonds.

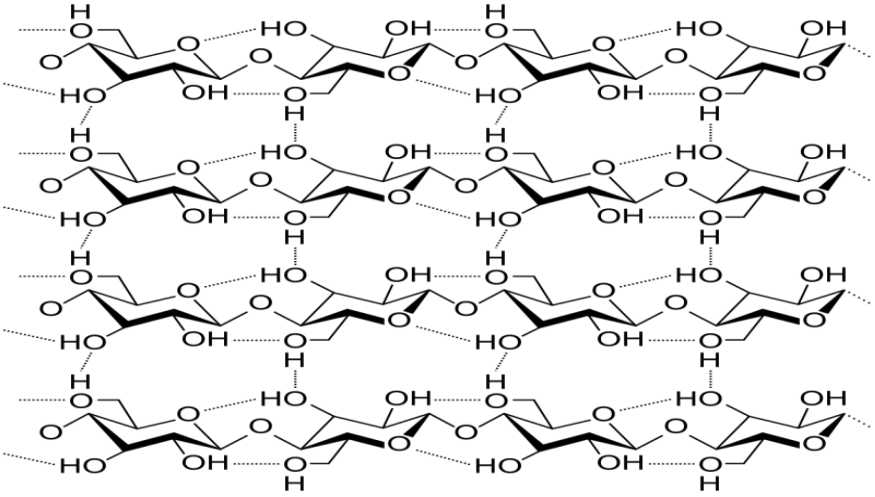

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Figure 3. A strand of cellulose (conformation Iα), showing the hydrogen bonds (dashed) within and between cellulose molecules.

1.1.3 Hemicellulose

Hemicellulose or polyose is one of the heteropolymers (polysaccharides backbone), for instance arabinoxylan, that is present in about all terrestrial plant cell walls along with cellulose. Generally cellulose is strong, crystalline, and tough to hydrolysis, but hemicelluloses do not have a unique structure, but are amorphous and have less strength (Scheller & Ulvskov, 2010). They are hydrolyzed simply by dilute bases or acids as well as by numerous enzymes (hemicellulases).

Hemicelluloses are made up of shorter chains than cellulose, containing about 500–3,000 sugar units, and they may be branched. They mediate binding between cellulose and pectin to form a cross-linked fiber network (Gibson, 2012).

Various types of sugar monomers along with glucose comprise hemicellulose. For instance, they contain the five carbon sugars arabinose and xylose, the six carbon sugars galactose and mannose, as well as the six carbon deoxy sugar rhamnose. Hemicelluloses comprise mostly of the D-pentose sugars, and small amounts of L-sugars occasionally. In most cases, xylose is the sugar monomer present in the largest amount, though mannose is the most abundant sugar in softwoods. Not only regular sugars are present in hemicellulose, but also acidic sugars, for

instance galacturonic acid and glucuronic acid can be found (Ebringerová et al., 2005; Heinze, 1958).

The biosynthesis of hemicelluloses occurs in the Golgi apparatus of cells. After successful biosynthesis of hemicelluloses, they are transported to the plasma membrane within vesicles. Specialized enzymes are responsible for each kind of hemicellulose biosynthesis (Zhu et al., 2019).

Some important hemicelluloses of plant cell wall are described briefly here:

1.1.3.1 Xylans

Xylans are present in the cell walls of plants; practically in all cell walls of grasses and the secondary cell walls of dicots. These are polysaccharides which are made up of pentose sugar namely β-1,4-linked xylose residues with side branches of α-glucuronic acids and α-arabinofuranose (Fig. 4). Xylans contribute to cross-linking of lignin and cellulose microfibrils through ferulic acid residues (Balakshin et al., 2011). According to the substituted groups, xylans are categorized into three groups i.e. glucuronoxylan, arabinoxylan and glucuronoarabinoxylan (Faik, 2010).

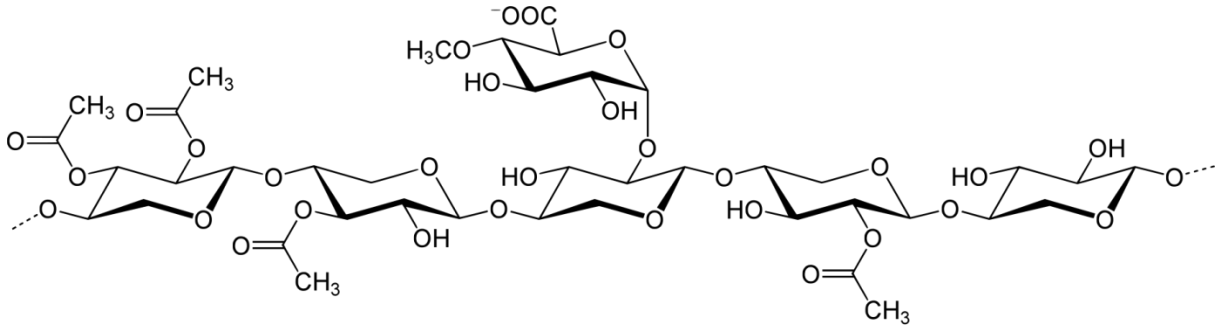

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Figure 4. Xylan in hardwood

1.1.3.2 Xyloglucan

Xyloglucan (one type of hemicellulose) exists in primary cell wall of the vascular plants. Xyloglucan is the most abundant hemicellulose found in the primary cell wall of many

dicotyledonous plants (Del-Bem, 2018). Xyloglucan anchors to the surface of microfibrils of cellulose and can bind them together. The backbone of xyloglucan consists of β (1→4)-linked glucose residues and it has 1→6 linked xylose side-chains (Fig. 5). Sometimes xylose residues are capped with galactose residue or by a fucose residue. The structure of xyloglucan varies among the plant families. The biosynthesis of xyloglucan occurs in Trans Golgi network and Golgi trans cisternae and then it is transported by vesicles to the cell membrane (Fry, 1989).

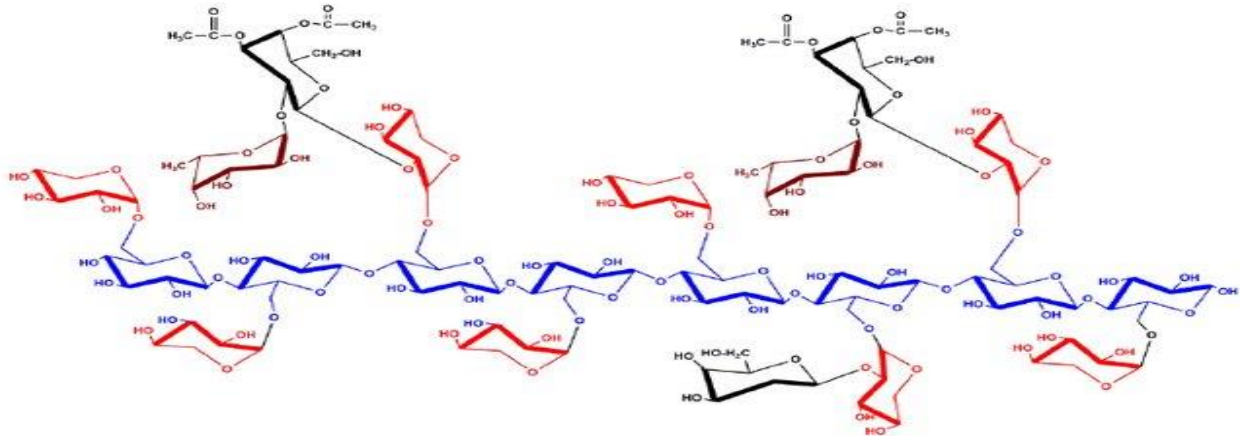

Figure 5. The structure of xyloglucan

1.1.3.3 Arabinoxylan

Arabinoxylan is one kind of hemicellulose present in both the primary as well as secondary cell walls of cereal grains and woody plants. It consists of two pentose sugars: xylose and arabinose. It is comprising of a linear backbone of β-1,4 xylose residues and many xylose units are xylose units are substituted with 2, 3 or 2,3-linked arabinose residues (Fig. 6). Three integral membrane enzymes belonging to glycosyl transferases (GT) are responsible to synthesize arabinoxylan and their side chains. Such as: arabinoxylan synthase, which generates the xylan backbone; arabinosyl transferases, which substitute arabinose for xylose residues; and arabinoxylan feruloyl transferase, that substitutes ferulate into arabinose residues (Mitchell et al., 2007). In cereal endosperm cell walls, where rye and wheat have the highest arabinoxylan content, arabinoxylan is abundant, followed by rice, barley, oats and maize. In plant cells, arabinoxylans play mainly a structural role. They are also the reservoirs of large quantities of ferulic acid and other phenolic

acids that are covalently bound to them. Some side-chains of arabinose carry esterified ferulic acid residues. As a consequence, via ferulic acid bridges, arabinoxylan chains in cell walls can be cross-linked with each other and/or cross-linked through ether or ester linkages with other cell-wall polymers (Wakabayashi et al., 2005).

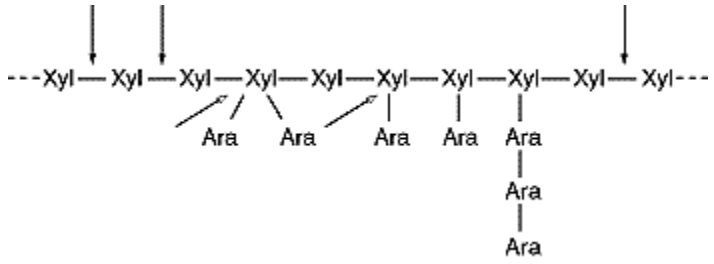

Figure 6. Chemical structure of arabinoxylan of barley

1.1.4 Pectins

Pectin was discovered by Henri Braconnot in 1825. It is a heteropolysaccharide acid. Its key component is galacturonic acid which is a sugar acid derived from galactose. Pectins are made up of a complex set of polysaccharides (Fig. 7). These are found mostly in primary cell walls and are predominantly abundant in the non-woody parts of terrestrial plants. Pectins are chief components of the middle lamella. They help to bind cells together, but they are also found in primary cell walls. Pectins are produced in Golgi and deposited into the cell wall via vesicles (Kepplar et al., 2006; Bidhendi et al., 2020).

The structure, amount and chemical composition of pectins varies among plants, in different parts of a plant and over time. Pectins are important polysaccharides in the plant cell wall and they allow plant growth and primary cell wall extension (Bidhendi et al., 2016). There are several types of pectin i.e. homogalacturonans (linear chains of α-(1–4)-linked D-galacturonic acid), rhamnogalacturonan I pectins (RG-I) (4-α-D-galacturonic acid-(1,2)-α-L-rhamnose-1), and rhamnogalacturonan II (RG-II) (backbone is made up of D-galacturonic acid units). The

molecular weight of isolated pectin is 60,000–130,000 g/mol, depending on origin and extraction methods (Buchanan et al., 2015; Albersheim et al., 2010).

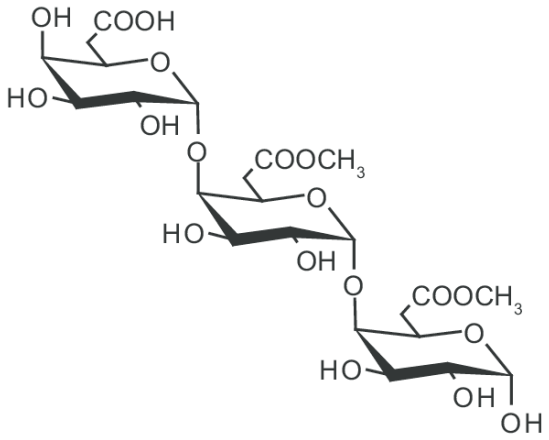

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Figure 7. The chemical structure of pectin

1.1.5 Proteins

The plant cell wall proteins play important roles in modifying the structure of wall and other components of cell wall, in interactions and signaling with proteins of the plasma membrane at the cell surface. The characteristics of cell wall proteins differ according to the functions of the various plant cell walls. Most of the cell wall proteins are cross-linked into the cell wall and may have structural roles (Jamet et al., 2006). They probably participate in morphogenesis also. For instance, arabinogalactan proteins are easily soluble and play an important role in cell-cell interactions in developmental events.

There are five types of protein in the cell walls i.e. 1) extensins, 2) glycine-rich proteins (GRPs), 3) proline-rich proteins (PRPs), 4) solanaceous lectins, and 5) arabinogalactan proteins (AGPs). These proteins are not the only cell wall proteins, there are some other types such as cysteine-rich thionins, histidine-tryptophan-rich proteins, and many other enzymes such as peroxidases, invertases, phosphatases, p-mannosidases, a-mannosidases, p1,3-glucanases, p1,4-glucanases, pectin methylesterases, polygalacturonase, arabinosidases, malate dehydrogenase, a-galactosidases, glucuronosidases, galactosidases, proteases, xylosidases, and ascorbic acid oxidase (Varner & Lin, 1989). Though, the above mentioned five classes of proteins generally represent the most abundant, and till now, the widely documented and most well-studied proteins of plant cell wall.

1.1.6 Other polysaccharides

The important polysaccharides of plant cell wall are described briefly here.

1.1.6.1 Xylogalacturonan

Xylogalacturonan (XGA) is a polysaccharide that contains galacturonic acid and xylose residues (Fig. 8). It is found in the plants cell walls. XGA was identified from an alkali extract of pine pollen. Chemically the xylogalacturonan comprised of homogalacturonan backbone with recurrent single xylose residues that linked β-(1→3) to almost half of the galacturonic acid residues (Mort et al., 2008).

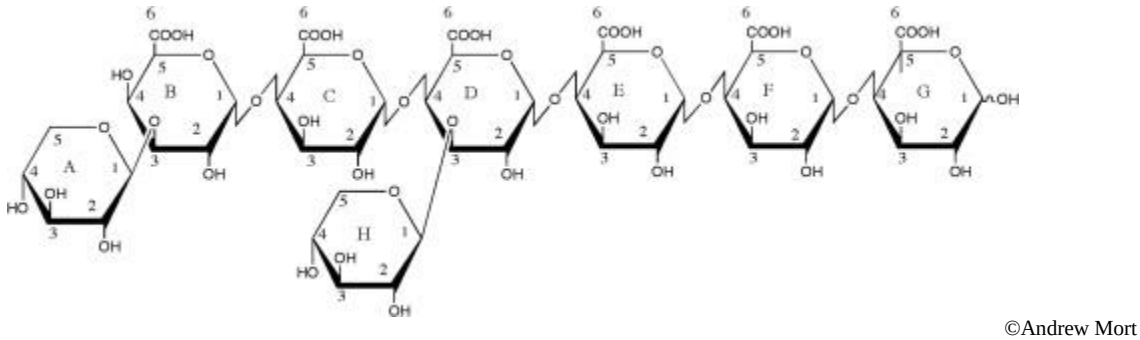

Figure 8. A structure of the xylogalacturonan

1.1.6.2 Homogalacturonan

Homogalacturonan is actually a linear homopolymer of 1,4-linked α-d-galacturonic acid and it is partly methyl-esterified at the C-6 carboxyl and may have other unidentified esters as well (Fig. 9). While values ranging from a degree of polymerization (DP) of 3022 to 2005 have been published, the duration of the HGA of pectin remains unsolved. Homogalacturonan (HG) is a key component of the middle lamella also found in primary walls of nongraminaceous monocots and dicots. HGs also present in secondary cell walls in minor quantities. HGs have cell adhesion, polyelectrolytic and ionic cross-linking properties (Sénéchal et al., 2014).

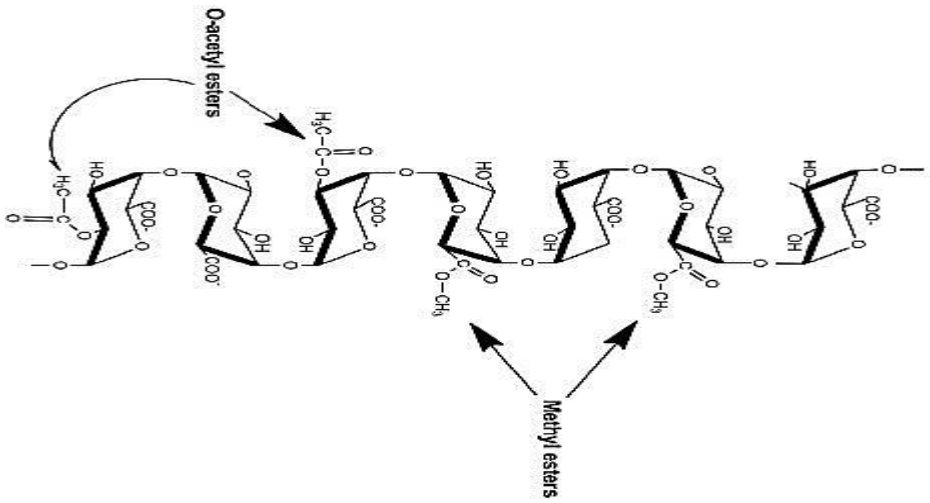

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Figure 9. The structure of homogalacturonan structure.

1.2 Norway spruce

The accepted name of Norway spruce is *Picea abies* (L.) H. Karst. Norway spruce is predominantly found in sub-Alpine coniferous forests and Europe's boreal regions, which ranges from East to Ural Mountains and Central to Northern Europe. Norway spruce is second most common tree species in Finland. There are two subspecies of Norway spruce found in Finland. The narrow crowned ssp. obovata dominates in the northern part, and the broader crowned ssp. abies dominates in the southern part. Hybrids of the two subspecies are also common. It grows generally from sea level in northern regions and to elevations of more than 2000 m in the Alps. Norway spruce has been cultivated since the 18th century. It is not only cultivated in its natural range but also widely in the British Isles (Tjoelker et al., 2007).

Norway spruce is a large coniferous tree. It can grow to the height of 50-60 m and develop more or less 150 cm in trunk diameter over a two to three hundred year life span. The crown is column or cone-shaped, with fine white-speckled lines, the needle-like leaves are light to dark green. They are 1 to 2.5 cm long, and have a cross section that is 4-angled. In early summer, the female

flowers open at the tip of the young shoots. At first, these are dark red, and then grow into 12-15 cm long cones that in the autumn turn green and then brown, and open up to disperse small-winged seeds (Lagercrantz & Ryman,1990).

Norway spruce is one of Europe's most significant coniferous plants, both economically and ecologically e.g. in paper pulp production and soil erosion control, respectively. Its timber is widely used in construction and in the production of paper, particularly in northern European countries. The wood is also used for a wide variety of items, such as for joinery, veneer, furniture and musical instruments (Hoffmeyer & Pedersen, 1995).

In 2013, the genome sequencing of Norway spruce was successfully accomplished (Nystedt et al., 2013). This is the first gymnosperm genome that has been completely sequenced. The genome comprises around 22 Gb (giga base pairs). This is about six times the size of the human genome but surprisingly possesses a similar number of genes. A huge proportion of this genome contains repetitive DNA sequences, and only 0.2% coding sequence. It includes long terminal repeats of transposable elements (Birol et al., 2013).

Spruce beer (made from the needles and buds of fresh Norway spruce shoots) was used to prevent scurvy (source of Vitamin C). Spruce tips are still used today by brewers to add to their beer a fresh and vibrant aroma. Many economically important chemical compounds i.e. cellulose, lignin, hemicelluloses, p-hydroxybenzoic acid glucoside, piceatannol and its glucoside (astringin), picein, isorhapontin (the isorhapontigenin glucoside), ferulic acid and catechin are found in Norway spruce (Münzenberger et al., 1990; Løkke, 1990).

1.2.1 Developmental biology of Norway spruce xylem

At the beginning of the growth season, trees produce long, thin-wall earlywood (EW) fibers and, later on, narrow and thick-wall latewood (LW) fibers. The cellulose and hemicellulose network of the cell wall in wood is impregnated with lignin, which sustains structural stability and facilitates the transport of water. There are several steps involved in the current view of the lignification process, beginning with the monolignol biosynthesis in the cytosol and then

transport through the plasma membrane. The transport mechanism of monolignol is still not only well characterized, but research results indicate that transporters named ATP-binding cassette (ABC) proteins play a very important role (Alejandro et al., 2012). Monolignols are exposed to extracellular laccases and peroxidases in the cell wall. These enzymes initiate lignin polymerization by oxidizing monolignols to phenolic radicals that pair non-enzymatically (Boerjan et al., 2003; Barros et al., 2015; Laitinen et al., 2017). Whether monolignol biosynthesis in conifers is a cell-autonomous process in xylem tracheids, the key form of cells in coniferous wood, or whether neighboring cells (i.e. ray parenchymal cells) are contributing to the development of monolignols, has been recently clarified. Cell culture experiments with the angiosperm species *Zinnia elegans* show that monolignols are supplied by non-differentiating cells to differentiating tracheal components (Hosokawa et al., 2001; Tokunaga et al., 2005; Pesquet et al., 2013).

Parenchymal cells and forming fibers in the xylem often produce monolignols for lignifying vessels in inflorescence stems of *Arabidopsis thaliana*, whereas lignification is a cell-autonomous process in inter-fascicular fibers (Smith et al., 2013, 2017). Based on staining with safranin and Alcian Blue it has been found that the ray parenchymal cells do not lignify in Norway spruce during the growing season.

Many studies have suggested that lignification and secondary cell wall formation occurs in the maturing xylem area. The xylem formation and development analysis by Gompertz function found that the highest rate of new cell division occurred at the beginning of July (5.6 cells per week) and that the overall number of cells in a fully developed growth ring was in average 63.5 (Panshin & de Zeeuw, 1980).

1.3 Lignification in plant cells

The biosynthesis of lignin occurs in apoplastic with multiple enzyme activities (Boerjan et al., 2003). The monolignols or lignin monomers named the hydroxycinnamyl alcohols coniferyl (CA), sinapyl (SA) and p-coumaryl (p-CA) alcohol are produced in the cytosol and then transported to the cell wall where they are bound together to make the lignin polymer.

In plants, lignin synthesis may be regulated developmentally or occurs as a response to biotic or abiotic stresses. During cell differentiation, lignin is deposited on the plant cell wall (Marjamaa et al., 2007). When lignification is caused by defence responses, it limits the spreading of invading pathogens (Menden et al., 2007).

The chemical structure and amount of lignin differs among plant species, different cell wall layers and cell types. Normally lignin content is higher in the coniferous wood (25-33%) than in the angiosperm wood (20-25%) (Adler, 1977). Lignin is predominantly comprised of guaiacyl (G) units synthesized from CA in conifers, whereas lignin is a co-polymer of CA and SA (guaiacyl (G) and syringyl (S) units) in angiosperms (Nimtz et al., 1981). H-units derived from p-coumaryl (p-CA) alcohol are existing in both gymnosperms and angiosperms in small amounts, but are ample in grasses (Nimtz et al., 1981). The lignin in grasses contains high amounts of hydroxycinnamyl acids, mostly ferulate and p-coumarate (Grabber et al., 1996; Ralph et al., 1998). The amount of lignin is higher in the middle lamella and cell corner than in the secondary walls (Agarwal, 2006; Gierlinger & Schwanninger, 2006). The G-units of lignin are more abundant in vessel cell walls than fibre walls of angiosperm trees. In the areas of cell corners and middle lamellae, H-type lignin is more abundant relative to the other cell wall layers (Fukushima & Terashima, 1991; Grünwald et al., 2002). Lignification is also influenced by changes in the environment; for example, compression wood formed on the lower side of conifer bent stems is characterized by higher lignin content and higher H-type lignin levels relative to regular wood (Önnerud & Gellerstedt, 2003). The cell wall development of xylem cells is one of the best examples of the lignification process. The water supplying cells of xylem tissue, vessel elements and tracheids are hollow dead cells. In tracheids, the cells are joined together with masses of ring pores and in vessels, openings at the vertical ends of cells. The tracheary elements undergo a very well-defined differentiation process involving specification, enlargement, patterned deposition of the cell wall, programmed death and removal of parts of the cell wall.

Cell wall lignification starts from cell corners and middle lamellae, following cell wall thickening by carbohydrate deposition, continuing through the secondary cell wall layers (Donaldson et al., 2001). The cellulose microfibrils and matrix carbohydrate polymers play important roles for lignin deposition, as shown in *Zinnia elegans* TEs treated with a cellulose

synthase inhibitor, which results in scattered lignification patterns (Taylor et al., 1992). Lignin is therefore shaped spherically in the loose carbohydrate network of the middle lamellae and the primary wall, while lignin forms elongated structures in the secondary wall with the strictly oriented cellulose microfibrils (Donaldson, 1994). In addition to developmentally regulated lignification (Hawkins & Boudet, 1996; Cabané et al., 2004). Plant-deposited stress lignins display structural differences from developmental lignins. For instance in wheat (*Triticum aestivum*) leaves lignins synthesized during a defense response were abnormally abundant with S-units (Menden et al., 2007). In an another example, elicitor-induced lignins in Norway spruce tissue culture were abundant with H-units (Cabané et al., 2004), and similar to early growing and compression wood lignins (Önnerud & Gellerstedt, 2003). The ozone-treated poplar trees store condensed lignins with high frequency of H-units (Lange et al., 1995).

1.3.1 Lignification in developing xylem

To establish whether parenchymal ray cells contribute to the biosynthesis of monolignols in the lignifying xylem of Norwegian spruce, a comparative transcriptomic study and a single-cell metabolome analysis were combined (*Picea abies*) (Blokhina et al., 2019). By supplying monolignols, ray parenchymal cells can act in the lignification of upright tracheids. Parenchymal ray cells and upright tracheids were dissected with laser-capture microdissection from tangential cryosections of spruce trees' xylem growth to test this hypothesis. It was found by transcriptome analysis that genes encoding cell wall biogenesis-related enzymes were highly expressed in both ray cells and developing tracheids. In addition, most of the pathway-related genes for shikimate and monolignol biosynthesis were similarly expressed in both types of cells (Blokhina et al., 2019).

Monolignols and their glycoconjugates were detected in in-situ single cell metabolomics of semi-intact plants by picoliter pressure probe-electrospray ionization-mass spectrometry in both cell types, suggesting that the biosynthetic route for monolignols is active in both parenchymal ray cells and upright tracheids (Blokhina et al., 2019). The research results support the hypothesis that ray cells generate monolignols that lead to the lignification of tracheid cell walls while developing xylem. Monolignols are found as free monolignols and monolignol glucosides in plants. It was suggested that monolignol glucosides, syringing, coniferin and p-coumaryl alcohol

glucoside should be either transport, storage or intermediate forms of monolignols (Steeves et al., 2001; Tsuji & Fukushima, 2004). It has been found that coniferin accumulation correlates spatially and temporally with the onset of secondary conifer growth (Freudenberg & Harkin, 1963; Savidge, 1989). Transport of monolignols/monolignol glucosides to the apoplast is thought to occur through specific ATP-binding cassette (ABC) transporters through Golgi mediated secretion or transport (Guillaumie et al., 2007).

2 Hypotheses and Objectives

The core objective of my thesis work is to discover the unknown step of lignification initiation step in the lignification process in developing xylem of Norway spruce. To achieve the goal, the aim is to investigate the chemical identity of lignification initiation sites in the middle lamellae and cell corners of developing Norway spruce xylem, to answer the question where in the cell wall soluble monolignols start the formation of lignin (polymerization). I am approaching this goal with immunolabeling technique and Raman spectroscopy to unravel this initiation site of lignification by using specific antibodies for cell wall compounds and comparing the results with the initial lignin deposition sites.

To analyze this, in thin sections of developing xylem, Raman spectroscopy was used in the present study to classify the chemical structure of the lignification regions. The use of monoclonal antibodies and immunomicroscopy to find ferulates and tyrosine residues of cell wall proteins in the cell wall was another method. We can obtain information about the initiation sites and their chemical composition by integrating these methods with image analysis.

I shall also address the issue of the fate of monolignols in the middle lamellae and cell corners and propose that in the middle lamellae, ferulic acid and probably other phenolics and tyrosine residues serve as an anchor compound for monolignols and, therefore, as lignification initiation sites. While ferulic acid is not an abundant constituent in the conifer cell wall, small amounts of ferulic acid in the primary cell wall of conifers are detectable with immunolocation (Carnachan

& Harris, 2000). In addition, a combination of factors such as pH, redox status, and a gradient of H_2O_2 can direct the start of lignin polymerization towards middle lamellae. For the first time in a coniferous tree, I expected to identify and characterize, to our knowledge, the identification of the biochemical environment in the lignification initiation sites, i.e. monolignol anchoring compounds will contribute to our understanding of the last steps in the formation of lignin that have not yet been identified.

My aim to investigate the chemical identity of lignification initiation sites in the middle lamellae and cell corners to answer the question of what forces monolignols to start the formation of lignin (polymerization) in the most proximal part of the cell wall, and far away from the plasma membrane.

At a glance, the objectives of my thesis are following:

- Identification of monolignol anchoring compounds in developing xylem of Norway spruce
  - Mapping the polysaccharides and lignin substructure distribution in developing xylem through antibody staining/immunolabeling
  - Mapping the monolignols distribution in developing xylem through Raman microscopy

3 Materials and Methods

Plant material was collected from three (03) mature (about 45 years old) Norway spruce tree clones in Ruotsinkylä forest area in southern Finland under the permission of the Natural Resources Institute Finland (LUKE).

3.1 Methods of sample preparation

The methods for immunolabeling (AIR, cryosections, MAbs mediated tissue treatment) and imaging (Confocal and Raman microscopy) are described below:

3.1.1 AIR procedure

The AIR (Alcohol Insoluble Residue) was prepared according to Fry (1988) for Comprehensive Microarray Polymer Profiling (CoMPP) (Chialva et al., 2019) analysis is as follows:

- The xylem material was snap frozen in liquid nitrogen. It was then homogenized with a blade grinder. The material was ground to a very fine powder.

After that homogenization, AIR was extracted as follows:

- 70% Ethanol was added and the sample vortexed thoroughly and span down at maximum speed for 10 min. EtOH was discarded.
- 1:1 Methanol and Chloroform were added and the sample vortexed thoroughly and span down at max speed for 10 minutes. Methanol and Chloroform were discarded.
- Acetone was added and the sample vortexed thoroughly and span down at maximum speed for 10 minutes. Acetone was discarded and the pellet was allowed to air dry.

Amounts used: For extraction 50-100 mg of air dried and homogenized material was extracted with 1.5 ml of solvents in the three extraction steps. The large volume was used to be sure to not saturate it with extractives released from the wood powder. A metal/glass ball was placed in the tube to make the re-suspension of the pellet easier.

After the material was dried it was weighed in eight-strip tubes (http://www.qiagen.com /products/catalog/lab-essentials-and-accessories/collection-microtubes#orderinginformation and http://www.qiagen.com/products/catalog/lab-essentials-and-accessories/collection-microtube-caps). Each tube contained around 10 mg of the AIR preparation. (Precise weight was recorded). Each tube was equal to one sample, and ten replicates were made.

3.1.2 Sample preparation for immunolabeling

The fresh cut blocks (three samples were used for each treatment) of Norway spruce stem (approx. 0.5 x 0.5 x 0.5 cm) were frozen on the cryomicrotome sample buttons with the help of a drop of water. Then the samples were allowed to freeze in the chamber of the cryomicrotome. Cross sections (14-18 µm) were cut using a sharp steel blade (Thermo Scientific HP35 Coated Microtome Blade). The samples were collected with a brush onto a glass slide containing a drop of distilled water. After two minutes, sufficient amount of cold 70% ethanol was pipetted on the top of the sections to fully cover the whole area. The slides were then transferred in sterile petri dishes and put into a laminar flow cabinet. Then ice cold 100% ethanol was used to substitute the 70% ethanol. This fixation step was performed at room temperature in the laminar flow cabinet. During the 2 minute incubation in ice-cold 100% ethanol, the cryosections were gently stretched and flattened with a pipette tip. The 100% ethanol was removed by pipetting and then the cryosections were allowed to dry for about 15 minutes. The slides with dry sections were collected into sterile 50 ml Falcon tubes. Then the tubes were tightly closed and stored at -20°C for not more than 7 days (Blokhina et al., 2017).

3.2 Immunolabeling with monoclonal antibodies against plant cell wall compounds

Moist chambers (12x12 cm square petri dishes) were prepared by putting wet filter paper on the sides of petri dish for incubation. Good quality ethanol-fixed sections were picked up for immunolabeling from -20°C and were allowed to equilibriate at room temperature. A hydrophobic barrier was drawn around the section (size ca. 1.2 x 1.2 cm) with a pen (Vector H-4000 Hydrophobic barrier pen). The area of this size required ca. 150 µl of liquid solution for incubating the section. Blocking was done using Phosphate buffer solution (PBS) pH7.5 and 1% non-fat milk protein in the moist chamber putting the petri dish on a slow tilting shaker for 1 hour. The primary antibodies (monoclonal antibodies) were diluted 1/10 (1/80 for DBD) in 1% MPBS. After blocking, sections were incubated with primary antibody at room temperature on the slow tilting shaker for 2 hours. Then the sections were washed with MPBS 3-5 times. After that, sections were incubated with the secondary antibody (dilution: 1/1000 and dilution: 1/200 only for Alexa fluor 633 of DBD) at 4°C overnight in the dark covered with aluminum foil

(Pedersen et al., 2012). The next day, the sections were washed with MPBS (3-5 times x 3 min) before the confocal microscopy.

Monoclonal antibodies (primary antibodies) against (1->4)-β-D-xylan (LM10), (1->4)-β-D-xylan / arabinoxylan (LM11), XXXG motif of xyloglucan (LM15), galactosylated xyloglucan (LM24), and Lignin substructure Dibenzodioxocin or DBD (kind gift of Prof. Teemu Teeri) were utilized in immunomicroscopy to study the presence of these epitopes in Norway spruce especially in the middle lamellae and cell corners where lignification of the cell wall starts (McCartney et al., 2005; Ruprecht et al., 2017; Pedersen et al., 2012).

The secondary antibodies containing the fluorescent label Alexa fluor 488 and 633 were used to identify the binding site of primary antibodies.

3.3 Confocal Microscopy

Cryomicrotome sections of developing xylem of Norway spruce treated with monoclonal antibodies and the secondary goat-anti-rabbit antibody were analyzed using confocal microscopy. Two types of confocal microscopes were used i.e. LEICA TCS SP5 II HCS-A and LEICA SP8 UPRIGHT for this study.

SP5II HCS is a completely motorized inverted point scanning confocal microscope. For dim samples and quicker imaging, the scanner has two extra sensitive low-noise detectors. For multipoint or tile imaging, the microscope has a motorized stage and the device has a Leica Matrix program for high-content plate imaging. Cross section snaps can also be acquired.

The SP8 Upright is a fully motorized Confocal microscope. It has four laser lines and three detectors, as well as the Leica Navigator motorized phase. This makes it possible to use the microscope to recreate 3D structures of both cells and tissue, but it appears to be slow due to the number of detectors imaging 3D samples with more than three colors. There is no enviromental control in this system and that's why it cannot be used with live samples that require CO_2 or heating. Notably the 25X 0.95 NA dipping water objective is excellent for large plant tissue samples, and it also lacks the high-magnification water objective.

The very specific primary antibodies for polysaccharides and the lignin substructure dibenzodioxocin were detected using secondary antibodies with excitation wavelength at (488 and 633 nm for Alexa fluor 488 and 633, respectively) and emission wavelength at (500-570 nm and 666-718 nm for Alexa fluor 488 and 633, respectively) by 20X objective, zoom 2.5-3.0.

3.4 Raman Microscopy

Cryomicrotome sections of developing xylem of Norway spruce were analyzed using Raman spectroscopy in collaboration with Assoc. Prof. N. Gierlinger in Vienna. This part of the work was kindly done by Assoc. Prof. Notburga Gierlinger at University of Vienna when Norway spruce samples sent to her. With this sensitive method, even small changes in lignin quantity and quality can be analyzed in xylem cell walls. Raman microscopy represents a nondestructive technique of imaging of plant cell walls that can provide understanding of the chemical composition with structures at the micrometer level (<0.5 μm). Multiple components can be analyzed within the native cell walls, and insights into polymer composition can be done (Gierlinger et al. 2012). There are three steps for imaging with Raman spectroscopy:

- **Sample preparation:** Cross sections (10-20 μm thick) of Norway spruce developing xylem was made with a cryomicrotome. Then the sections were mounted with heavy water (D_2O) on glass slides under a coverslip and sealed with nail varnish.
- **Spectra acquisition:** For spectra acquisition with a confocal Raman microscope the Lasers, spectrometer, CCD-camera, objective and motorized scan table were optimized for high light throughput. The tens of thousands of spectra of one single map represent position-resolved molecular fingerprints.
- **Data analysis:** To do an adequate data analysis spectral pre-treatment i.e. cosmic ray removal (CRR), baseline correction and smoothing etc. was done. Imaging (visualization) was done by integration of band intensity and cluster analysis. The calculation of chemical images was done on the basis of the acquired Raman spectra. Every Raman image was based on thousands of spectra, each of them being a spatially resolved molecular 'fingerprint' of the cell wall.

4 Results

The results obtained from confocal and Raman microscopies are described below.

4.1 Confocal microscopy

The 14-18 μm thick cryomicrotome sections of Norway spruce developing xylem were viewed and photographed under the confocal microscope with appropriate wavelengths as explained in Materials and Methods. The monoclonal antibodies (MAbs) against cell wall bound polysaccharides and a lignin substructure were seen to bind clear parts of the cell wall, and hence the outcome of the confocal microscopy is promising. Here the results are described according to the monoclonal antibodies used.

4.1.1 Detection of xylan with LM 10 antibody

The monoclonal antibody LM10 binds with (1->4)-β-D-xylan molecules of plant cells. The xylan contributes to cross-linking of lignin and cellulose microfibrils through ferulic acid residues. Hence, xylans can be considered very important in lignification. The locations of xylans in Norway spruce xylem cells were investigated through applying LM10 on the thin wood sections. The images below show that xylans are present in the secondary cell wall abundantly, and some is present in the primary cell walls also. Antibody staining is found not only in cell corners but also evenly in the whole cut cell wall surface. There is some highly dense signal coming out from ray cells. We know that ray cells contain various kinds of molecules which could produce the signals seen in Fig. 10 A and C. The control samples partially support the accuracy of the monoclonal antibody treatment as no signals were found from ray cells of the samples.

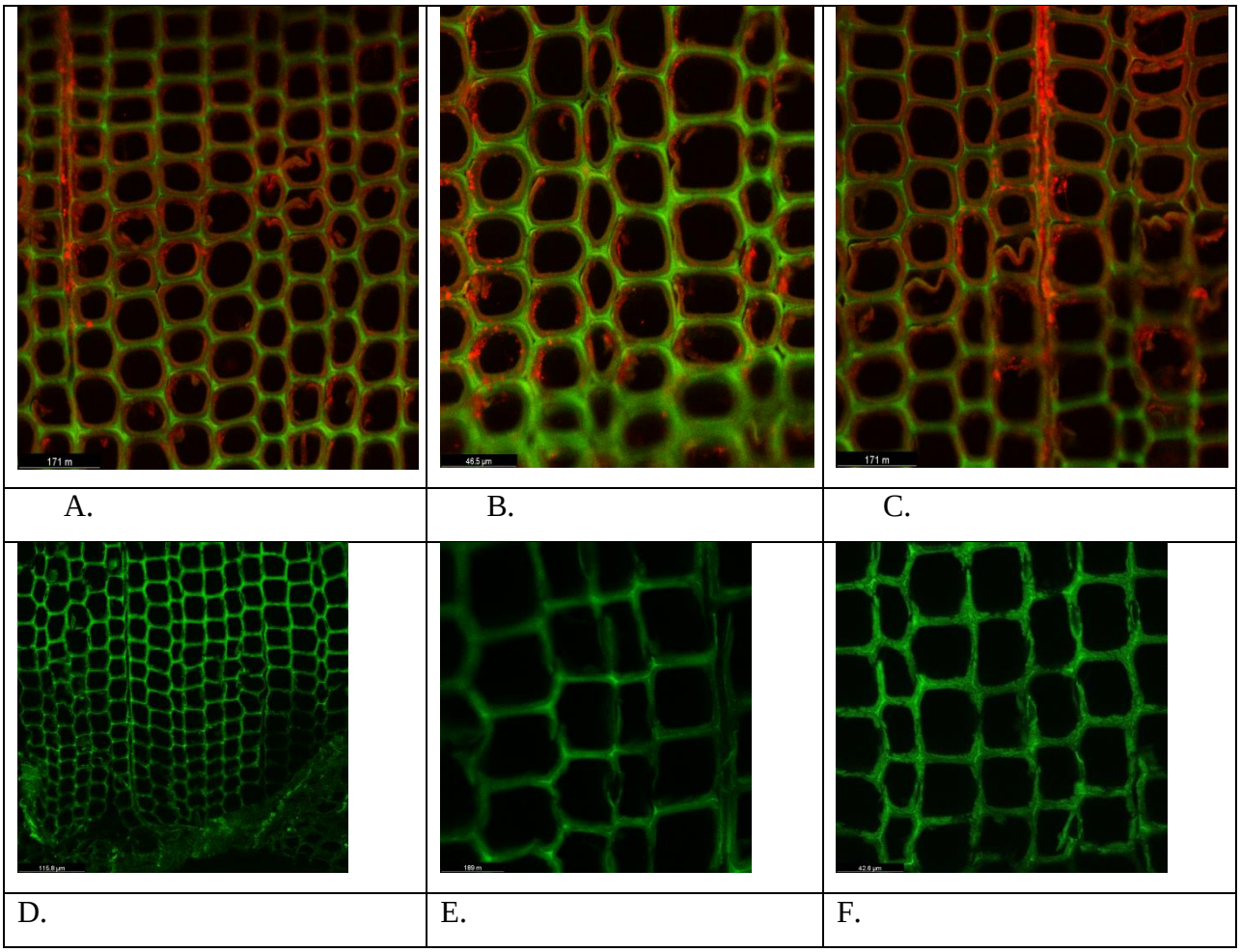

Figure 10 (A-E). Photomicrographs from A to C show the samples treated with both primary (LM10) and secondary (Alexafluor-633) antibodies. From D to F show the controls i.e. in D, LM10 is absent but Al-633 is present but in E, the treatment is opposite and in F, both primary and secondary antibodies are absent. The red colored signal is coming from the primary antibody (LM10) bound with the secondary antibody labeled with Alexafluor-633. The green color signal is from lignin auto-fluorescence.

4.1.2 Detection of arabinoxylan and unsubstituted xylans with LM11 antibody

The arabinoxylan and (1->4)-β-D-xylan (unsubstituted xylans) are the reservoirs of huge amounts of ferulic acid and other phenolic acids. These phenolic acids and ferulic acids are covalently linked to arabinoxylan. The monoclonal antibody LM11 binds with arabinoxylan and unsubstituted xylans of plant cells. hence, we can expect that arabinoxylan and

unsubstituted xylans may play important roles in lignification. The location of xylans in Norway spruce xylem cells were explored through using LM11. The images below show that arabinoxylan and unsubstituted xylans are present in primary cell wall abundantly and some are found in secondary cell walls also. These are abundant in newly formed cells and less in more developed cells. The controls are supporting the accuracy of the monoclonal antibody treatment as no specific (red) signal is seen in any of the control samples in Fig. 11. Though we expected some auto-fluoroscence signal from the ray cells of controls.

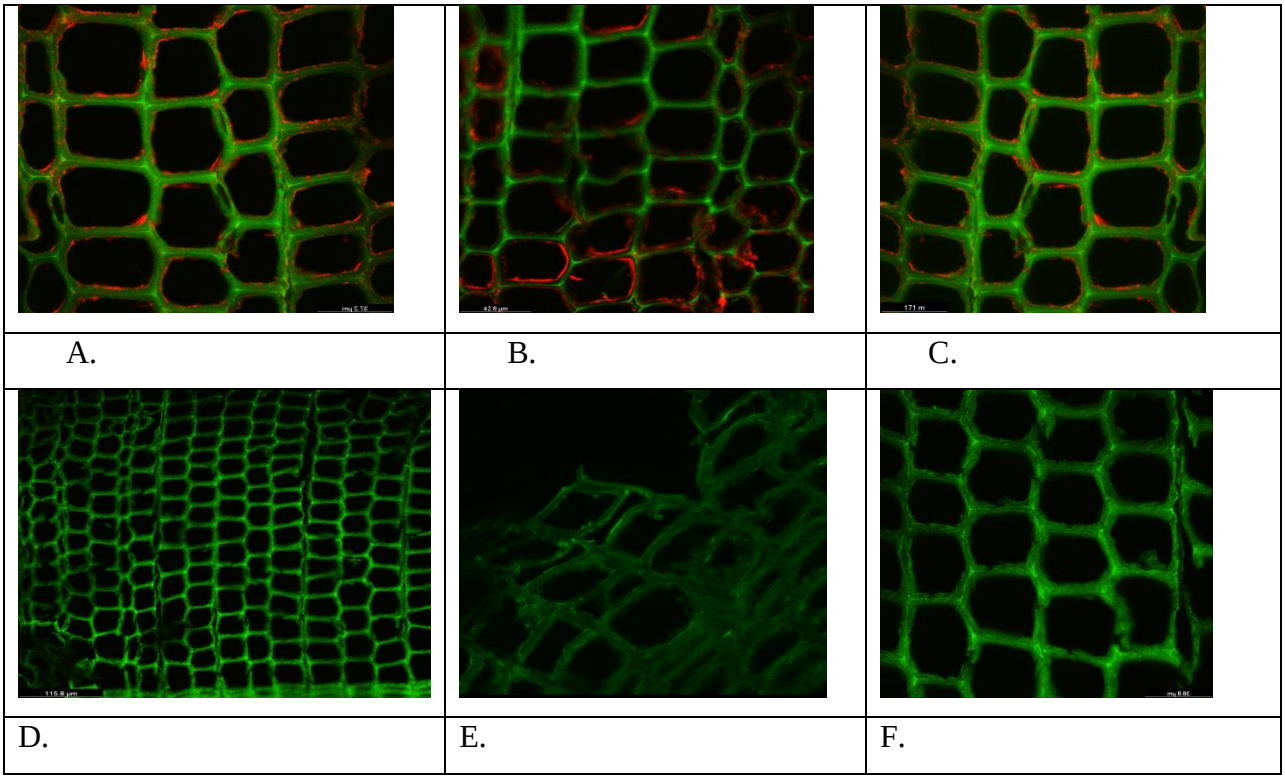

Figure 11 (A-E). Photomicrographs from A to C show the samples treated with both primary (LM11) and secondary (Alexafluor-488) antibodies. From D to F show the controls i.e. in D, LM11 is absent but Al-488 is present but in E, the treatment is vice-versa and in F, both primary and secondary antibodies are absent. The red colored signal is coming from the primary antibody (LM11) bound with the secondary antibody labeled with Alexafluor-488. The green color signal is from lignin auto-fluorescence.

4.1.3 Detection of XXXG motif in xyloglucan with LM15 antibody

LM15 monoclonal antibody detects the location of XXXG motif of xyloglucan in plant cells. Actually, xyloglucan binds to the surface of cellulose microfibrils and could link them together. Xyloglucan was located in the middle lamellae, and primary and secondary cell wall of Norway spruce, as shown in images given below (Fig. 12). The images of the control samples (Figs 12. D-E) missing one, two or both primary and secondary antibodies did not show any specific signal as expected.

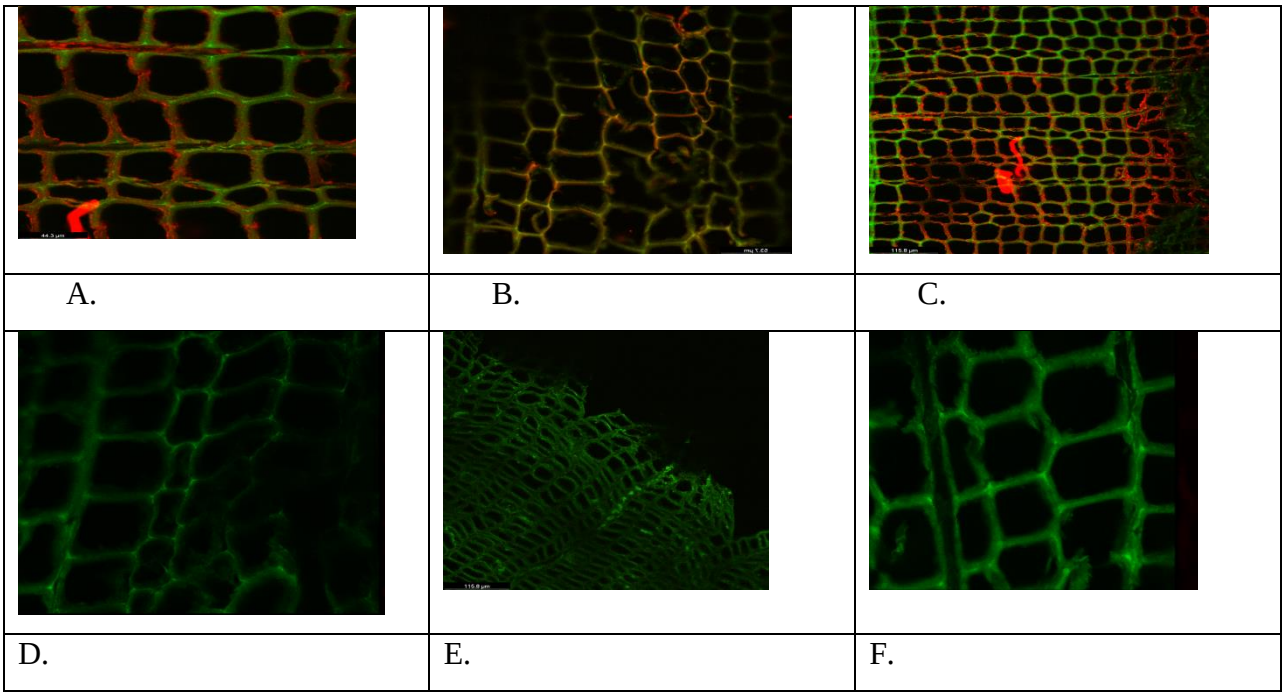

Figure 12 (A-E). Photomicrographs from A to C show the samples treated with both primary (LM15) and secondary (Alexafluor-488) antibodies. From D to F show the controls i.e. in D, LM15 is absent but Al-488 is present but in E, the treatment is vice-versa and in F, both primary and secondary antibodies are absent. The red colored signal is coming from the primary antibody (LM15) bound with the secondary antibody labeled with Alexafluor-488. The green color signal is from lignin auto-fluorescence.

4.1.4 Detection of galactosylated xyloglucan with LM24 antibody

The monoclonal antibody LM24 was produced against galactosylated xyloglucan of plant cells. Normally, xyloglucan binds to the surface of cellulose microfibrils and can link them together. The images in Fig. 13 show the location of xyloglucan in the secondary cell walls. One of the controls is showing signal which may confer the red signal may be from some autofluroscent compound in this section.

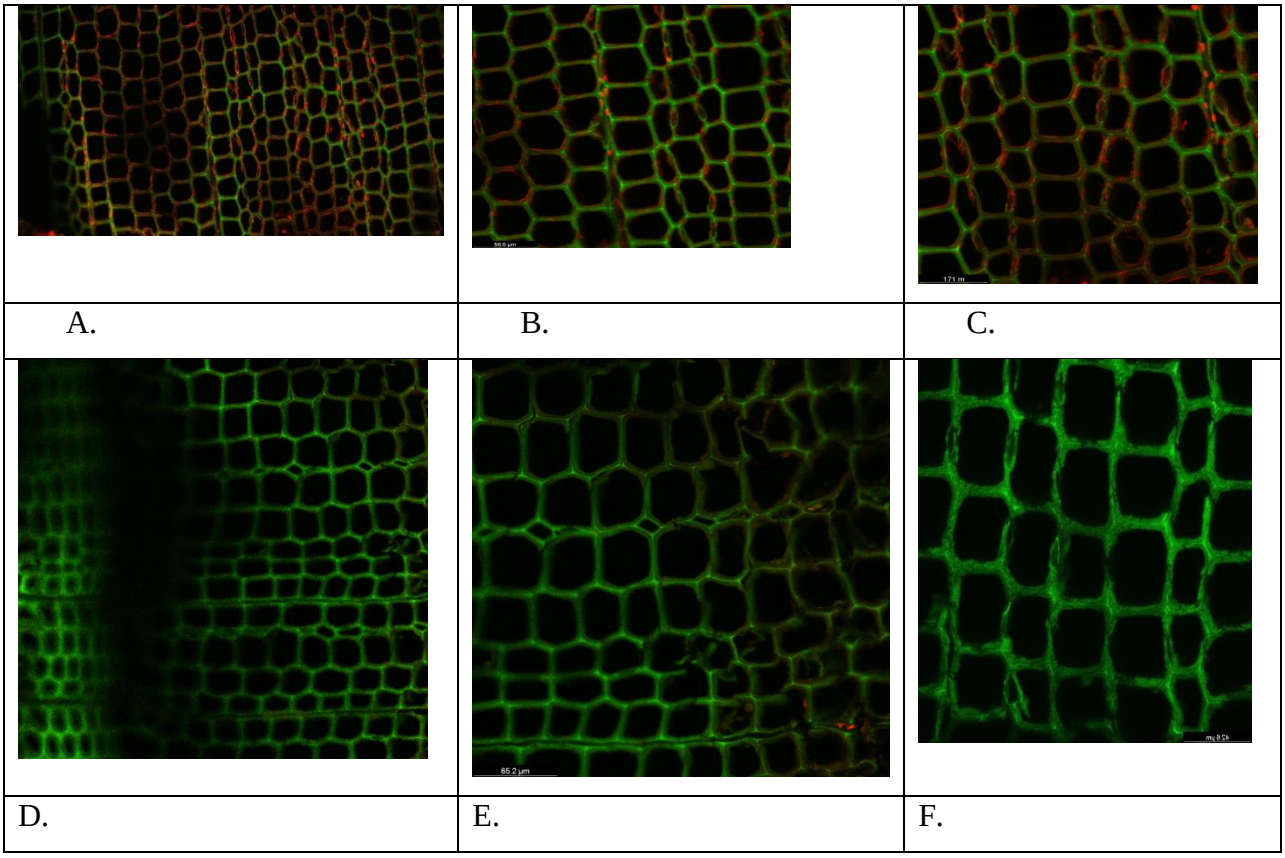

Figure 13 (A-E). Photomicrographs from A to C show the samples treated with both primary (LM24) and secondary (Alexafluor-633) antibodies. From D to F show the controls i.e. in D, LM24 is absent but Al-633 is present but in E, the treatment is opposite and in F, both primary and secondary antibodies are absent. The red colored signal is coming from the primary antibody (LM24) bound with the secondary antibody labeled with Alexafluor-633. The green color signal is from lignin auto-fluorescence.

4.1.5 Detection of dibenzodioxocin (DBD) with a rabbit antibody

The lignin substructure was determined by dibenzodioxocin or DBD antibody produced in rabbits (kind gift of Prof. Teemu Teeri). This antibody was used as a control to test the whole staining procedure. This antibody has been used earlier to detect DBD in the xylem of many Finnish tree species. The images in Fig. 14 show that this structure is located in the mature cells at secondary cell walls. The cells which do not yet have the secondary cell wall, this lignin substructure is absent.

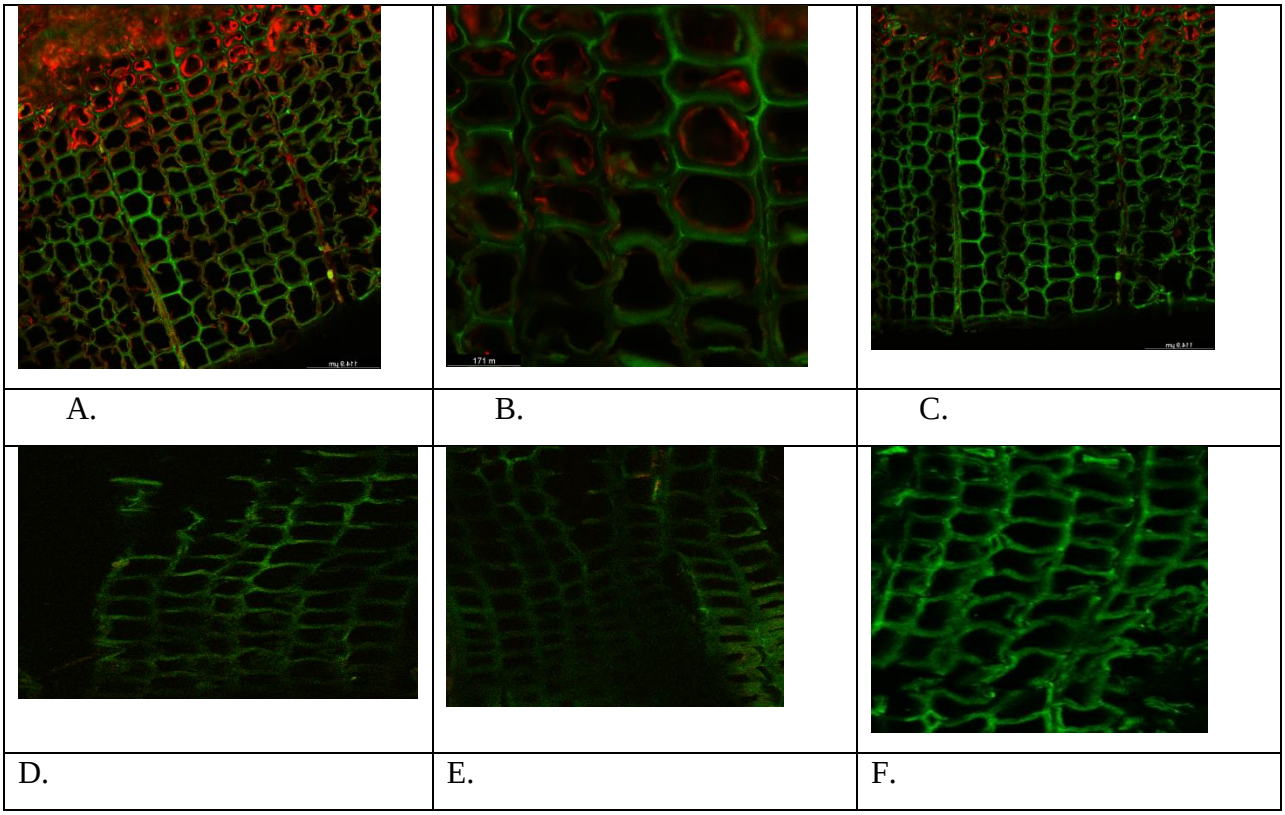

Figure 14 (A-E). Photomicrographs from A to C show the samples treated with both primary (DBD) and secondary (Alexafluor-633) antibodies. From D to F show the controls i.e. in D, DBD is absent but Al-633 is present but in E, the treatment is opposite and in F, both primary and secondary antibodies are absent. The red colored signal is coming from the primary antibody (LM10) bound with the secondary antibody labeled with Alexafluor-633. The green color signal is from lignin auto-fluorescence.

4.2 Raman microscopy

The developing xylem of Norway spruce was investigated through Raman microscopy technique to identify the lignification initiation site (Fig. 15). This part of the work was kindly done by Assoc. Prof. Notburga Gierlinger at University of Vienna. The Norway spruce samples were sent to her. The first image (inset 1) is the microscopic view of Norway spruce xylem with cambial region and phloem. The second image (inset 2) shows the developing Norway spruce xylem; the red border area was analyzed by Raman microscopy. The inset images from 3 to 8 were captured by Raman microscopy with various spectral wave numbers. The yellowish/golden color indicates that the molecules/compounds are present in that certain area and dark/black color denotes the absence of them. Cinnamyl alcohol, a monolignol was determined using 998 nm spectrum, and it was abundantly found at cell corners and middle lamellae in most developing part of xylem (inset 6). Coniferyl alcohol, one of the monolignols, was detected at cell corners only in developing xylem of Norway spruce by 1660 nm spectrum (inset 7). These molecules are absent in the edge cells very near the cambium in the developing xylem. Coniferyl aldehyde, one kind of monolignol, was identified at cell corners, middle lamellae and primary cell walls (inset 8) of Norway spruce developing xylem. Coniferyl aldehyde was found more at the developing part than in the mature part of the xylem.

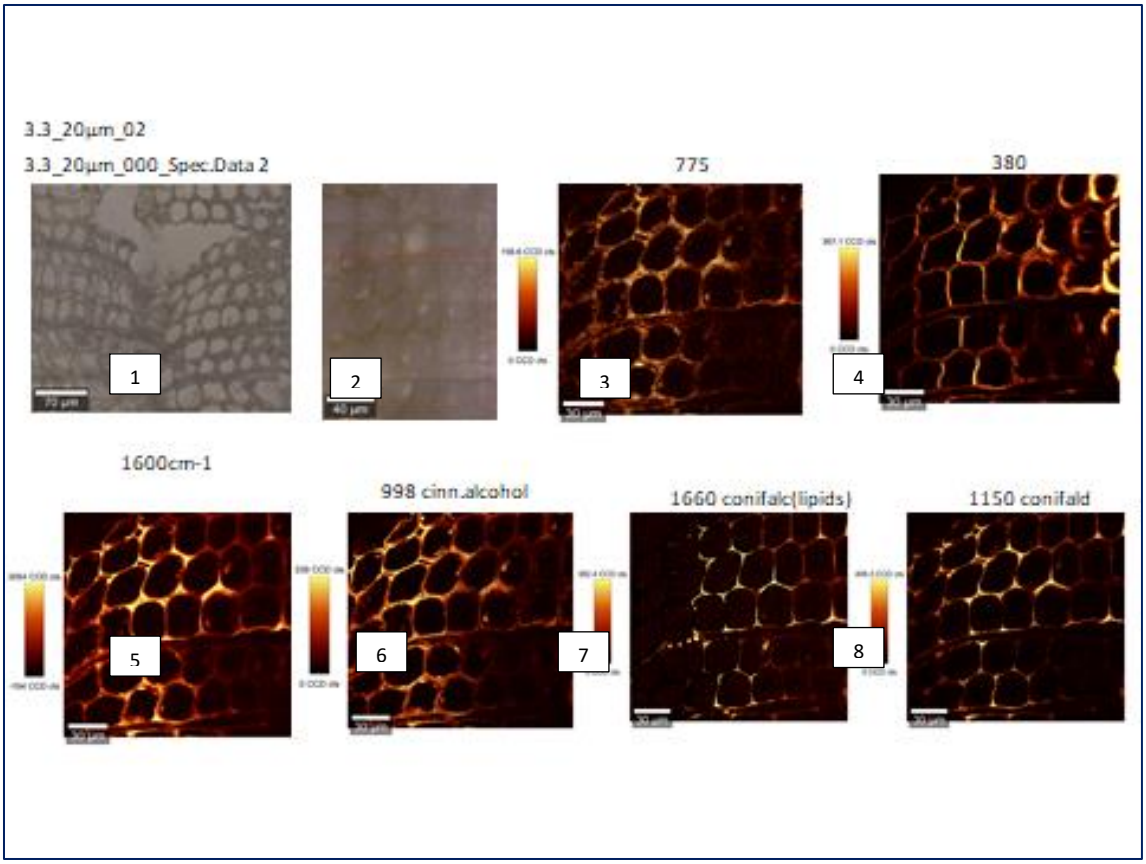

Figure 15 (inset 1-8). Developing xylem analysis with Raman Microscopy. **Inset 1** indicates the microscopic view of Norway spruce xylem with cambial region and phloem. **Inset 2** indicates the developing part (youngest) of Norway spruce xylem. Inset 3 to 8 were captured by Raman microscopy with various spectral wave numbers. The yellowish/golden color indicates that the molecules/compounds are present in that certain area and dark/black color denotes the absence of them. **Inset 6,** Cinnamyl alcohol, a monolignol was determined using 998 nm spectrum. **Inset 7**, Coniferyl alcohol, one of the monolignols, was detected at cell corners only in developing xylem of Norway spruce by 1660 nm spectrum. **Inset 8**, Coniferyl aldehyde was identified at cell corners, middle lamellae and primary cell walls of Norway spruce developing xylem by 1150 nm spectrum. N.B. These images were taken by Assoc. Prof. Notburga Gierlinger.

5 Discussion

I was looking for any potentially active residues in the cell wall, middle lamellae and cell corners that could act as initiation sites for lignification. Such active groups could be e.g. galacturonan in pectins and ferulic acid residues hemicelluloses that could react with mono- or oligolignols. Free tyrosine residues in cell wall proteins are another possibility: the hydrophobicity of tyrosine residues creates a favourable environment for lignin monomer coupling while the hydrophilic OH-group may participate in the electron transfer reactions (Ralph & Lu, 2010). To achieve the goal, immunolabeling and Raman microscopy approaches were applied.

Cell wall specific polysaccharides and a lignin substructure were observed by monoclonal antibodies (MAbs) through confocal microscopy. The LM10 monoclonal antibody binds with xylan of plant cell wall. Xylan is favorably found in the S1-S2 transition zone, and it contributes to cross-linking of cellulose microfibrils and lignin through ferulic acid residues (Awano et al., 2002). In this study, LM10 signals were detected in secondary cell wall abundantly and some in in primary cell walls also. The epitopes are present not only in cell corners but also evenly in whole cell wall surface of Norway spruce.

The LM11 monoclonal antibody binds with arabinoxylan in addition to unsubstituted xylans, which can store large amounts of ferulic acid (McCartney et al., 2005) as well as other phenolic acids which are covalently bound to them (Rao & Muralikrishna, 2006). Generally, arabinoxylan is present in both the primary and secondary cell walls of plants (McCartney et al., 2005). In this study, LM11 helped in the detection of arabinoxylan and unsubstituted xylans that were present in the primary cell wall abundantly, and som signal was also found in the secondary cell walls. Arabionoxylans are plentiful in new cells and less in matured ones.

The LM15 monoclonal antibody is against xyloglucan. Xylogulan binds to the surface of cellulose microfibrils and holds them together. Normally xyloglucan is present in the primary walls, middle lamellae and the gelatinous wall layer (reaction wood) of woody plants (Pramod et al., 2019). In this study, xyloglucan was located in the middle lamellae, and primary and secondary cell walls of Norway spruce.

The LM24 monoclonal antibody binds with glycosylated xyloglucan, which is linked to cellulose microfibrils. These are generally found in the primary walls, middle lamellae and gelatinous wall layers in reaction wood of woody plants (Pramod et al., 2019). In our study, this antibody was

found to bind in secondary cell walls and abundantly in cell corners. Some signal was located in the primary cell wall. Sometimes, the controls were showing signals in ray cells which may confer unspecific binding of the secondary antibody with ray cell walls or some other compounds.

The Dibenzodioxocin or DBD monoclonal antibody binds to a lignin substructure which is found in the S3 layer of the secondary wall in both Norway spruce and silver birch (Kukkola et al. 2003). In our study, the signal was located in the mature cells of the secondary cell walls (S2 and S3 layers). This lignin substructure is absent in cells which do not have the secondary cell wall.

Raman imaging of developing xylem of Norway spruce reveals the location of monolignols i.e. cinnamyl alcohol, coniferyl alcohol and coniferyl aldehyde. The cinnamyl alcohol was abundantly found at cell corners and middle lamellae in the developing part of xylem with the 998 nm spectrum. Chabannes et al., (2001) found that both Cinnamyl alcohol dehydrogenase (CAD) and Cinnamoyl CoA reductase (CCR) down-regulated plants had less/disturbed lignification in S2 sublayer. Coniferyl alcohol was located at cell corners only in developing xylem of Norway spruce as seen in the 1660 nm spectrum. Coniferyl alcohol was not found in the youngest region of the developing xylem. The very preliminary first RAMAN images indicated that this technique could be used the detection of initial lignification of Norway spruce xylem. According to Morikawa et al., (2010), coniferin (a glucoside of coniferyl alcohol) was identified during the S2 layer-forming stage in the cell corner middle lamellae of *Chamaecyparis obtusa*. The Raman images of Hänninen et al., (2011) revealed that higher amount of coniferyl alcohol is present in secondary cell wall than in the primary cell wall and middle lamella area. Higher amounts of coniferyl aldehyde groups are found in the primary cell wall and middle lamellae of spruce. In this study, coniferyl aldehyde was determined at cell corners, middle lamellaee and primary cell walls of developing xylem. Coniferyl aldehyde was found less in mature cells than in younger ones.

The comparative image analysis between immunolabeling and Raman microscopy reveals that the monolignols i.e. coniferyl alcohol are present in the cell corners, cell wall layers and middle lamellae of the developing xylem where the xylan, arabinoxylan, xyloglucan and lignin substructures DBD are available. So, it indicates the monolignols could anchor to those polysaccharides and then lignin formation initiates.

6 Conclusions

The confocal and Raman microscopy approaches to identify the probable lignification initiation site(s) in developing xylem of Norway spruce was successful in identifying the distribution of polysaccharides, monolignols and a lignin substructure in Norway spruce cell walls. The information of the immunolabeling and Raman images paves way to map the process of lignification in Norway spruce developing xylem. These information will help to conduct research in molecular biology level (gene knockouts) for identification of the genomic and proteomic interaction network for lignification of Norway spruce xylem in future.

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